

ORIGINAL**Follicular fluid 8-Hydroxy-2-Deoxyguanosine (8-OHdG) as biomarker for oxidative stress in intracytoplasmic sperm injection**Maher Abbood Mukheef^{1, 4}, Rihab Abbas Ali^{2, 5}, and Hanaa Hameed Abbas Alheidery³

¹University of Kerbala / College of medicine / Department of biochemistry, Kerbala, Iraq. ORCID Number : 0000-0002-8787-4522, ²University of Kerbala / College of medicine / Department of anatomy, histology and embryology, Kerbala, Iraq. ORCID Number : 0000-0003-1614-9144, ³Jabir ibn hayyan medical university / College of medicine / Department of Obstetrics and Gynecology, Al-Najaf, Iraq, ⁴University of Warith Al-Anbiyaa / College of medicine / Department of biochemistry, Kerbala, Iraq, ⁵University of Warith Al-Anbiyaa / College of medicine / Department of anatomy, Kerbala, Iraq

Abstract : Oxidative stress (OS) is a situation that occurs as a result of un-equilibrium between reactive oxygen species (ROS) formation and the antioxidant defence system. 8-hydroxy-2-deoxyguanosine (8-OHdG), an oxidized form of deoxyguanosine, is found in higher levels in aging oocytes. In this cross-sectional study, we aimed to evaluate the effect of 8-OHdG on the intracytoplasmic sperm injection (ICSI). Follicular fluid (FF) samples were taken after removing the oocyte for later analysis of 8-OHdG. The couples participants were categorized according to the cause of subfertility into three groups (female factor, malefactor, and unexplained infertility). Further division according to pregnancy state was done to evaluate the precise role of 8-OHdG on pregnancy state and ICSI outcome. **Result :** This study showed that the 8-OHdG levels significantly higher in non-pregnant women ($p < 0.05$). Correlation study showed that 8-OHdG level in follicular fluid is negatively correlating with the number of retrieved oocytes, metaphase two oocytes (MII), fertilized oocytes (2PN=two pronuclei), cleaved zygotes, good quality embryos, and the difference was statistically significant ($p < 0.05$). **Conclusion :** The study revealed that the 8-OHdG level in follicular fluid negatively influences ICSI outcome and it is higher in non-pregnant women. *J. Med. Invest.* 69:112-116, February, 2022

Keywords : 8-OHdG, ROS, ICSI, oxidative stress

INTRODUCTION

Subfertility is the failure to get pregnant despite having regular and unprotected (non-contraceptive) intra-vaginal sexual relationships for 12 months (1, 2). Oxidative stress (OS) is a situation that results from un-equilibrium between reactive oxygen species (ROS) formation and the antioxidant defense system. At the proper level, it may mediate some physiological functions, but an excessive amount will promote the development of a pathological process that negatively influences reproductive function (3, 4). Follicular fluid (FF) plays a fundamental role in the oocyte performance as the FF provides an environment for oocyte development, so any imbalance between antioxidant systems and ROS in the FF may be resulting in abnormal development of the oocyte, which causes the damage of the cytoskeleton, deoxyribonucleic acid (DNA), and cell membrane. This intimately affects oocytes quality and subsequent embryonic development (5). Strong evidence suggests that poor oocyte quality increased DNA damage, accredited to excess ROS generation. 8-hydroxy-deoxyguanosine (8-OHdG), an oxidized form of deoxyguanosine. 8-OHdG is the commonest base alteration in mutagenic damage and acts as an OS biomarker (6). Oxidants from different metabolic activity, toxins, radiation, or inflammation can destruct nucleic acids producing lesions that seem to contribute to ageing and various reproductive disease that impair

fecundity (7). Moreover, it is considered a factor in promoting and initiating carcinogenesis (8).

The hydroxyl radical ($\text{HO}\cdot$) interact with the nucleobases in the DNA strand, like guanine, which results in the C8-hydroxyguanine (8-OHGua) production or when interacting with its nucleoside (guanosine) form deoxyguanosine (8-hydroxy-2-deoxyguanosine). ROS attacks the 8th carbon atom of guanine in the DNA to create 8-OHDG (which is an oxidized derivation of deoxyguanosine), which is found with higher levels in aging oocytes (9).

The study is designed to verify the precise roles of follicular fluid 8-OHdG and how it can affect the ICSI outcomes.

PATIENTS AND METHOD

An analytic cross-sectional study was done in the fertility centre, Al-Sadr Medical City, Al-Najaf governorate, Iraq. It included sub-fertile couples ($n=88$) who attended the fertility center to deal with their fertility problem by ICSI from June 2021 to September 2021. The procedure was explained to the couples, and all selected couples' informed consent was taken before involving them in the study. Furthermore, the procedure steps were explained to and approved by the ethical committee of the University of Kerbala College of Medicine. 88 infertile couples were selected randomly and divided into three groups according to the cause of subfertility (female factor, malefactor and unexplained subfertility). Female patients age range is 18-40 years old, body mass index (BMI) range 18.5-30 kg/m^2 and male partners with either normal or mild to moderate malefactors were included in this study. Male partners with severe male factors, female partners with diabetes or other endocrine diseases such

Received for publication December 19, 2021 ; accepted January 30, 2022.

Address correspondence and reprint requests to Rihab Abbas Ali, University of Kerbala/College of medicine/Department of anatomy, histology and embryology, Kerbala, Iraq.

as thyroid disease, and females with current untreated pelvic pathology like moderate-to-severe endometriosis, submucosal uterine fibroids, Asherman's syndrome, uterine malformations, and hydrosalpinx all were excluded.

Sample size was calculated according to the following equation for cross sectional study with quantitative variables (10) :

$$\text{Sample size} = (Z_{1-\alpha/2})^2 \text{SD}^2/d^2$$

$Z_{1-\alpha/2}$ = is standard normal variate = 1.96

SD = standard deviation of variable. The value of SD can be taken from previous done study.

d = absolute error or precision as determined by the researcher.

Sample size = $(1.96)^2 (0.4)^2 / (0.1)^2 = (3.84 * 0.16) / 0.01 = 61$ patients.

So the sample size is 61 patients, our study involved 88 patients.

Regarding female partners, medical, gynecological, and surgical history were reviewed. Also, complete physical examination and anthropometric measures (height, weight, and BMI). On cycle day 2 (CD2), vaginal ultrasound (U/S) was done to determine the antral follicle counts, ovarian condition, and endometrial thickness. The level of serum follicle stimulation hormone (FSH), luteinizing hormone (LH), and estradiol (E2) all were measured on CD2 by enzyme-linked immunosorbent assay (ELISA) kit (Immuno-tech-Beckman Coulter, Webster, TX, USA) and expressed in pg/ml, IU/ml respectively.

Male partners were assessed by history, physical examination, and seminal fluid analysis. According to WHO criteria, the male partners included in the study were either normal semen parameters or mild to moderate male factor subfertility according to WHO criteria 2010.

Ovarian stimulation protocols : After full evaluation and examination, the female partners were enrolled through ovarian stimulation protocols, either the agonist or antagonist protocol, according to the patient's age, BMI, and cause of infertility.

For the agonist protocol, the patients were included in a short protocol (n=56). It started on day 2 of the cycle by the subcutaneous (s.c) administration of 0.1 mg/day of Triptorelin (decapeptyl) (for pituitary down-regulation), which is gonadotropin-releasing hormone (GnRH) agonist and lasted till human chorionic gonadotropin (hCG) injection day. On day 3 of menses, recombinant FSH (Gonal-f, Merck Serono Specialities Pvt. Ltd., Italy) was added by daily s.c injection to stimulate the ovary and was stopped one day before hCG administration. While the antagonist protocol (n=32), we used the flexible regimen, which was started on day 2 or 3 of menstruation by gonadotropin injection for ovarian stimulation. Recombinant FSH daily s.c injection was given (Gonal-f, Merck Serono Specialities Pvt. Ltd., Italy) and lasted until the hCG administration day. Then estradiol (E2) levels were more than 500 pg/ml, and the diameter of the prominent follicle was achieved 14-15 mm, cetorelix (cetrotide) 0.25 mg (Merck Serono, Italy) injected s.c. which is GnRH antagonist. Their injection continued until there were minimally three follicles ≥ 17 mm in diameter, then hCG was given.

Ovulation Triggering and Ova Pick up : When the level of estradiol > 1500 p/ml and there is minimally three follicles ≥ 17 mm in diameter. Ovulation triggering is achieved either by the administration of hCG (Pregnyl) 10000 IU or GnRH agonist (Decapeptyl 0.2 mg) S.C The ova pick-up was carried out under general anaesthesia, guided by transvaginal ultrasound 36 hours after ovulation trigger. The good-quality metaphase two (MII) oocytes were injected by sperms and incubated in special conditions. Samples of FF were taken at the time of oocytes pick up to measure 8-OHdG. Fertilization assessment was done 16-18

hour after injection to see the two pronuclei (2PN). On day 2 or 3 post-injection, the quality and number of the embryos were assessed, and no more than three top-quality embryos were transferred to the uterus. Extra embryos were either discarded or cryopreserved. The number of follicles, collected oocytes, embryos number and quality, number of transferred embryos, and pregnancy rate all were studied and statistically analyzed.

Sample of the FF is taken (After scanning for oocyte) and centrifuged at 3000 rounds per minute (rpm) for 10 minutes ; then the supernatant was collected and divided up in multiple Eppendorf safe-lock tubes and store at -20°C for later use.

8-OHdG (MT) ELISA Assays : Elisa Kit (Elabscience, USA) was used to determine 8-OHdG concentrations in FF quantitatively by the competitive-ELISA principle.

STATISTICAL ANALYSIS

Patient data were presented and statistically analyzed using two software programs : Microsoft office excel 2010 and Statistical Package for Social Sciences (SPSS version 22.00). Categorical data were expressed as number and percentage, whereas, Numeric, quantitative variables were expressed as mean and standard deviation $M \pm SD$, continuous variables between the two groups were compared using an independent t-test. At the same time, the estimation of the differences of means among multiple groups was done using one-way ANOVA. The Chi-square test was used to evaluate the differences between groups in categorical variables. Bivariate analysis (Pearson's correlation test) was used to examine the degree of correlation between measured parameters and other clinical parameters. $P \leq 0.05$ was considered as statistically significant (Daniel, 1999)

RESULTS

The mean age of the females was 30.29 ± 5.82 years (range 18-40 years), while the mean BMI was 24.63 ± 2.58 (kg/m^2).

The patients were grouped according to the aetiology. It was found that 29.54 % (26/88) of cases have female factor, while 45.54% (40/88) of points have male factor subfertility. For the remaining 25.00% (22/88) of patients, no apparent cause was identified, referred to as unexplained infertility. The female factor was further subdivided into polycystic ovarian syndrome (PCOS), which accounts for 46.15% (12/26) ; tubal blockage for 34.61% (9/26) ; and 19.23% (5/26) due to mild endometriosis.

In this study, the percentage of primary subfertility among total cases was 73.87% (65/88), while those who suffered from secondary subfertility were 26.13% (23/88).

There were two types of ovarian stimulation protocols used in this study ; short agonist protocol used in 38.6% (34) of cases and antagonist protocol in 61.36% (54/88) of cases. In addition, 72.72% (64/88) of the patient underwent ICSI for the first time, while 27.27% (24/88) had previous attempts. Hormonal profiles on cycle day two (CD2) were represented as follows : mean \pm SD : For LH = 3.92 ± 2.5 (mIU/ml). FSH = 6.34 ± 2.82 (mIU/ml) and E2 = 34.29 ± 13.58 (pg/ml). The overall biochemical pregnancy rate in this study was 27.27% (24/88). While the clinical pregnancy rate was 22.59% (19/88).

Table (1) shows a comparison among the three studied groups regarding age, BMI, basal hormonal profile, pregnancy rate and the follicular levels of 8-OHdG on the day of oocyte pick-up. The pregnancy rate is calculated per cycle (per ET). The data are expressed in mean \pm SD using the One Way ANOVA test for the numerical variables and in percentage and frequency for pregnancy rate using the Chi-square test. The level of serum FSH

is significantly higher in the group of unexplained infertility. There is a statistically significant difference in the follicular fluid 8-OHdG among the different causes of infertility, with the highest level in the female factor (P-value <0.05).

Table 1. Comparing basal finding among different group of infertility.

Variable	Female factor (n=26)	Male factor (n=40)	Unexplained (n=22)	P-value
Age years	30.96±6.77	30.18±5.77	29.73±5.56	0.782
BMI (kg/m ²)	25.33±2.56	23.94±2.32	25.08±2.83	0.066
Basal E2 (pg/ml)	31.92±17.87	38.18±10.52	30.04±11.14	0.091
Basal FSH (mIU/ml)	5.93±2.73	5.69±2.47	8.03±2.97	0.004*
Basal LH (mIU/ml)	4.14±3.62	3.74±1.98	3.99±1.74	0.112
8-OHdG(ng/mL)	2.42±0.88	1.83±0.49	1.89±0.55	0.001*
Pregnancy rate %	23.03%	30.00%	27.27%	0.827

BMI = body mass index, E2 = estrogen, FSH = follicle stimulation hormone, LH = luteinizing hormone, 8-OHdG = 8-hydroxy-2-deoxyguanosine.

*= P value <0.05 and it is statistically significant.

Table (2) compares the level of 8-OHdG between the two groups (pregnant and non-pregnant) by independent sample t-test. As shown in the table, the level of 8-OHdG in the FF of pregnant women is lower than the non-pregnant women ; the difference was highly statistically significant (p < 0.05).

Table 2. Follicular fluid 8-OHdG and pregnancy state.

Variable	Pregnant (n=24)	Non-pregnant (n=64)	p-value
8-OHdG (ng/mL)	1.50±0.41	2.21±0.63	0.000006

8-OHdG = 8-hydroxy-2-deoxyguanosine.

Table (3) there was a statistically significant negative correlation between FF 8-OHdG on the day of pick-up and the number of oocytes retrieved, MII, 2PN, total number of the embryo, GII, GIII embryos. Also, the table shows a significant negative correlation between maternal age and the number of mature oocytes, MII. On the other hand, maternal age correlates positively and statistically significantly with the level of the FF 8-OHdG.

While GI and GIV embryos, LH, E2, also correlate with 8-OHdG negatively, the difference was not statistically significant. Although 8-OHdG correlates positively with FSH, GV, fertilization rate, and cleavage rate, the difference was not statistically significant.

DISCUSSION

The present study highlights the association between the level of 8-OHdG with the outcome of ICSI at oocyte retrieval day to evaluate the local role of these substances in reproductive physiology. It also discusses the role of maternal age in the success of ICSI, how the 8-OHdG is affected by maternal age, and how its role reflects the ICSI outcome. The ICSI outcomes include (number of follicle, number of retrieved oocytes, number of injected MII, number of the fertilized oocyte (2PN), number and quality of embryos, fertilization rate, cleavage rate, number of transferred embryos, and lastly, the biochemical and clinical pregnancy rate).

This study examined the oxidative state in various subfertility causes and evaluated the oxidative state between pregnant and

Table 3. Correlation between follicular fluid 8-OHdG and age with the hormonal profile and ICSI outcomes.

Variable		8-OHdG	Age
Oocytes number	r	-0.22	-0.133
	p	0.04*	0.216
GV	r	0.001	0.232
	p	0.9	0.030*
MII	r	-0.267	-0.227
	p	0.012*	0.033*
2PN	r	-0.224	-0.154
	p	0.036*	0.151
Embryos number	r	-0.222	-0.155
	p	0.038*	0.149
GI	r	-0.133	-0.153
	p	0.2	0.155
GII	r	-0.264	-0.104
	p	0.013*	0.333
GIII	r	-0.249	-0.040
	p	0.02*	0.710
GIV	r	-0.09	0.014
	p	0.4	0.896
Fertilization rate	r	0.009	0.140
	p	0.9	0.193
Cleavage rate	r	0.03	0.054
	p	0.7	0.619
FSH	r	0.1	-0.119
	p	0.3	0.269
LH	r	-0.087	-0.010
	p	0.4	0.924
E2	r	-0.2	-0.133
	p	0.059	0.216
8-OHdG	r		0.275
	p		0.010*

*. Correlation is significant at the 0.05 level (2-tailed).

8-OHdG = 8-hydroxy-2-deoxyguanosine, GV = germinal vesicle, MII = metaphase two oocytes, 2PN = two pronuclei, GI = grade one, GII = grade two, GIII = grade 3, GIV = grade four, FSH = follicle stimulation hormone, LH = luteinizing hormone, E2 = estradiol.

non-pregnant women. Analysis showed a clinically significant difference in the level of 8-OHdG in the FF between the different causes of subfertility, with the highest level being in the female factor infertility ; this explains the theory that excess ROS may hamper the uterine endometrium that maintains and support the embryo development. Also, many female reproductive system pathologies such as polycystic ovary syndrome, endometriosis, preeclampsia, tubal obstruction and recurrent abortions are related to the presence of high levels of free radicals that may damage biological molecules such as lipids, proteins, or DNA (11-13). Likewise, Hosen *et al.* mentioned that the sperm DNA damage resulting from oxidative stress could critically affect the etiology of infertility in males (14).

Our results showed that the FF 8-OHdG is significantly higher in non-pregnant women, attributed to the ROS effect that induces programmed cell death, causing embryo fragmentation, implantation failure, miscarriage, and hereditary abnormalities

(15). Interestingly, Singh *et al.* agreed with our finding as they mentioned that increased ROS levels were related to pregnancy failure (16). Lastly, ROS may induce regression of corpus luteum, causing inadequate luteal hormones necessary to sustain pregnancy (9).

The current study confirmed that 8-OHdG is negatively correlated with the number and maturity state of oocytes, fertilized oocytes, the total number of embryos, and good quality embryos, in statistically significant differences. Studies demonstrated the pathological role of excess ROS concentration and its negative impact on ICSI outcome. They exhibited that 8-OHdG level in granulosa cells is inversely associated with oocyte quality and embryo development in ICSI program (17). Additionally higher 8-OHdG level in FF is associated with a higher number of degenerated oocytes, signifying the toxic effect of 8-OHdG on the oocyte in the follicular compartment.

Our result is in accordance with previous literature, which shows the negative influence of rising FF ROS levels on oocyte development and embryo quality (5, 18). Elizur *et al.* study mentioned that top-quality embryos are inversely linked to ROS concentrations in the FF (19). Oxidative stress affects the female reproductive system and induces subfertility via different mechanisms, mainly through direct damage to the gametes when the follicle contains excess ROS that overcomes the physiological antioxidant defence system, causing direct damage to the oocytes and spermatozoa DNA, which may impair fertilization (14).

Maternal age is a cornerstone factor affecting the ovulation induction program during the preparation for the ICSI procedure. Maternal age substantially impacts the oocyte quality and ovarian reserve and subsequently the number and quality of collected oocytes during the ICSI program (20). Our study showed a significant negative correlation between maternal age and the number of collected mature oocytes (MII). This finding agrees with previous studies that mentioned a significant reduction in the number and quality of collected oocytes with increased age of the female partner (20, 21). However, the level of 8-OHdG in our study had significant negative correlation with the number of mature oocytes from one side and in significant positive correlation with increased maternal age on the other side, so this could be an explanation why is the negative impact of maternal age on the quality and number of the oocyte.

The main limitation of the study was the difficulty in sample collection besides the limited funding resources. A cross-sectional study is another limiting factor since this study design will not elucidate the long term effect of 8-OHdG on female fertility potential nor have prediction power (22). For that, we recommend a cases control study to explore the association of 8-OHdG with fertility potential in optimal prediction values.

Oxidative stress role is acknowledged in many gynecological and obstetrical diseases (11, 12, 23). Many pursued novel biomarkers for diagnostic and therapeutic application; this study emphasizes the impact of oxidative stress on the ICSI outcome and how ROS can negatively affect fertility. Inability to get pregnancy or poor ICSI outcome due to oxidative stress is a treatable condition and should be addressed to improve fertility potential and outcome before enrolling in the ART program. Further studies are recommended to reverse the effect of 8-OHdG (24, 25) in couples fertility potential.

CONCLUSION

Oxidative stress can remarkably affect fertility in females. Therefore, ROS could result in poor ICSI outcomes and failed pregnancies. For cases with low pregnancy rates, ROS such as 8-OHdG should be excluded.

CONFLICT OF INTEREST

On behalf of all authors, the corresponding author states that there is no conflict of interest.

ACKNOWLEDGEMENTS

We acknowledge all medical and paramedical staff who worked in the IVF Center, Iraq, Al-Najaf.

REFERENCES

1. Starc A, Trampuš M, Pavan Jukić D, Grgas-Bile C, Jukić T, Polona Mivšek A: Infertility and sexual dysfunctions: a systematic literature review. *Acta Clin Croat* 58: 508-515, 2019. <https://doi.org/10.20471/acc.2019.58.03.15>
2. Ali RA, Al-Murshidi SYH, Al-jarah DM: The Role of Serum and Follicular IL-1Beta in Predicting the ICSI Outcome in Infertile Women. *Indian J Public Health Res & Development* 10: 511, 2019. <http://dx.doi.org/10.5958/0976-5506.2019.00100.1>
3. H Sekhon L, Gupta S, Kim Y, Agarwal A: Female infertility and antioxidants. *Current Women's Health Reviews* 6: 84-95, 2010. <http://dx.doi.org/10.2174/157340410791321381>
4. Adeoye O, Olawumi J, Opeyemi A, Christiania O: Review on the role of glutathione on oxidative stress and infertility. *JBRA Assist Reprod* 22: 61, 2018. <https://doi.org/10.5935/1518-0557.20180003>
5. Jana SK, Babu N, Chattopadhyay R, Chakravarty B, Chaudhury K: Upper control limit of reactive oxygen species in follicular fluid beyond which viable embryo formation is not favorable. *Reprod Toxicol* 29: 447-451, 2010. <https://doi.org/10.1016/j.reprotox.2010.04.002>
6. Salmon AB, Richardson A, Pérez VI: Update on the oxidative stress theory of aging: does oxidative stress play a role in aging or healthy aging?. *Free Radic Biol Med* 48: 642-655, 2010. <https://doi.org/10.1016/j.freeradbiomed.2009.12.015>
7. Ji LL, Peterson DM: Aging, exercise, and phytochemicals promises and pitfalls. *Ann N Y Acad Sc* 1019: 453-461, 2004. <https://doi.org/10.1196/annals.1297.083>
8. Valavanidis A, Vlachogianni T, Fiotakis C: 8-hydroxy-2'-deoxyguanosine (8-OHdG): a critical biomarker of oxidative stress and carcinogenesis. *J Environ Sci Health C Environ Carcinog Ecotoxicol Rev* 27: 120-139, 2009. <https://doi.org/10.1080/10590500902885684>
9. Tamura H, Takasaki A, Miwa I, Taniguchi K, Maekawa R, Asada H, Sugino N: Oxidative stress impairs oocyte quality and melatonin protects oocytes from free radical damage and improves fertilization rate. *J Pineal Res* 44: 280-287, 2008. <https://doi.org/10.1111/j.1600-079x.2007.00524.x>
10. Charan J, Biswas T: How to calculate sample size for different study designs in medical research?. *Indian J Psychol Med* 35: 121, 2013. <https://dx.doi.org/10.4103%2F0253-7176.116232>
11. Iborra A, Palacio JR, Martínez P: Oxidative stress and autoimmune response in the infertile woman. *Chem Immunol Allergy* 88:150-162, 2005. <https://doi.org/10.1159/000087832>
12. Badr Roomi A, Nori W, Mokram Hamed R: Lower Serum Irisin Levels Are Associated with Increased Osteoporosis and Oxidative Stress in Postmenopausal. *Rep Biochem Mol Biol* 10: 13-19, 2021. <https://doi.org/10.52547/rbmb.10.1.13>
13. Nori W, Ali AI: Maternal alpha-1-antitrypsin as a novel marker for growth restriction in pre-eclampsia. *J Obstet*

- Gynaecol Res 2021. <https://doi.org/10.1111/jog.15043>
14. Hosen MB, Islam MR, Begum F, Kabir Y, Howlader MZH : Oxidative stress induced sperm DNA damage, a possible reason for male infertility. *Iran J Reprod Med* 13 : 525, 2015
 15. Agarwal A, Said TM, Bedaiwy MA, Banerjee J, Alvarez JG : Oxidative stress in an assisted reproductive techniques setting. *Fertil Steril* 86 : 503-512, 2006. <https://doi.org/10.1016/j.fertnstert.2006.02.088>.
 16. Singh AK, Chattopadhyay R, Chakravarty B, Chaudhury K : Markers of oxidative stress in follicular fluid of women with endometriosis and tubal infertility undergoing IVF. *Reprod Toxicol* 42 : 116-124, 2013. <https://doi.org/10.1016/j.reprotox.2013.08.005>.
 17. Agarwal A, Allamaneni SS : Role of free radicals in female reproductive diseases and assisted reproduction. *Reprod Biomed Online* 9 : 338-347, 2004. [https://doi.org/10.1016/s1472-6483\(10\)62151-7](https://doi.org/10.1016/s1472-6483(10)62151-7).
 18. Borowiecka M, Wojsiat J, Polac I, Radwan M, Radwan P, Zbikowska HM : Oxidative stress markers in follicular fluid of women undergoing in vitro fertilization and embryo transfer. *Syst Biol Reprod Med* 58 : 301-305, 2012. <https://doi.org/10.3109/19396368.2012.701367>.
 19. Elizur SE, Lebovitz O, Orvieto R, Dor J, Zan-Bar T : Reactive oxygen species in follicular fluid may serve as biochemical markers to determine ovarian aging and follicular metabolic age. *Gynecol Endocrinol* 30 : 705-707, 2014. <https://doi.org/10.3109/09513590.2014.924100>.
 20. Mukheef MA, Ali RA, Majeed HH : How Can the Maternal Age and the Basal Level of Serum FSH Predict the ICSI Outcome?. *Indian Journal of Forensic Medicine & Toxicology* 15 : 1695, 2021
 21. Zahir RAl-yasiry, H AA, Hasan MF, Alkafaji NN : The Impact of Maternal Age on Intracytoplasmic Sperm Injection (ICSI) Outcomes in Infertile Couples. *Medico Legal Update* 20 : 822-826, 2020. <https://doi.org/10.37506/mlu.v20i4.1925>.
 22. R. C. Solem : Limitation of a cross-sectional study. *Am J Orthod Dentofacial Orthop* 148 : 205, 2015. <https://doi.org/10.1016/j.ajodo.2015.05.006>
 23. Yoshida K, Kusama K, Fukushima Y, Ohmaru-Nakanishi T, Kato K, Tamura K : Alpha-1 antitrypsin-induced endoplasmic reticulum stress promotes invasion by extravillous trophoblasts. *Int J Mol Sci* 22 : 3683, 2021. <https://doi.org/10.3390/ijms22073683>
 24. Eryilmaz OG, Devran A, Sarikaya E, Aksakal FN, Mollamahmutoğlu L, Cicek N : Melatonin improves the oocyte and the embryo in IVF patients with sleep disturbances but does not improve the sleeping problems. *J Assist Reprod Genet* 28 : 815-820, 2011. <https://doi.org/10.1007/s10815-011-9604-y>.
 25. Liu RH, Li YH, Jiao LH, Wang XN, Wang H, Wang WH : Extracellular and intracellular factors affecting nuclear and cytoplasmic maturation of porcine oocytes collected from different sizes of follicles. *Zygote* 10 : 253-260, 2002. <https://doi.org/10.1017/s0967199402002332>.